Biomedicine Key Laboratory of Shaanxi Province[1],Northwest University, Xi'an; National Institute for the Control of Pharmaceutical and Biological Products[2], Beijing, China; Institut de Chimie Moléculaire (FR 2769)[3], Laboratoire de Chimie Organique (UMR CNRS 7611), Université Pierre & Marie Curie-Paris 6, Paris, France

# Qualitative and quantitative analysis of the diuretic component ergone in *Polyporus umbellatus* by HPLC with fluorescence detection and HPLC-APCI-MS/MS

YING-YONG ZHAO[1], YE ZHAO[1], YONG-MIN ZHANG[3], RUI-CHAO LIN[1, 2], WEN-JI SUN[1]

*Received December 6, 2008, accepted December 12, 2008*

*Prof. Wen-Ji Sun, Biomedicine Key Laboratory of Shaanxi Province, Northwest University, No.229 Taibai North Road, Xi'an 710069, China*
*cxbml@nwu.edu.cn*

*Pharmazie 64: 366–370 (2009)* *doi: 10.1691/ph.2009.8364*

*Polyporus umbellatus* is a widely used anti-aldosteronic diuretic in Traditional Chinese medicine (TCM). A new, sensitive and selective high-performance liquid chromatography-fluorescence detector (HPLC-FLD) and high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS/MS) method for quantitative and qualitative determination of ergosta-4,6,8(14),22-tetraen-3-one(ergone), which is the main diuretic component, was provided for quality control of *P. umbellatus* crude drug. The ergone in the ethanolic extract of *P. umbellatus* was unambiguously characterized by HPLC-APCI, and further confirmed by comparing with a standard compound. The trace ergone was detected by the sensitive and selective HPLC-FLD. Linearity ($r^2 > 0.9998$) and recoveries of low, medium and high concentration (100.5%, 100.2% and 100.4%) were consistent with the experimental criteria. The limit of detection (LOD) of ergone was around 0.2 μg/mL. Our results indicated that the content of ergone in *P. umbellatus* varied significantly from habitat to habitat with contents ranging from $2.13 \pm 0.02$ to $59.17 \pm 0.05$ μg/g. Comparison among HPLC-FLD and HPLC-UV or HPLC-APCI-MS/MS demonstrated that the HPLC-FLD and HPLC-APCI-MS/MS methods gave similar quantitative results for the selected herb samples, the HPLC-UV methods gave lower quantitative results than HPLC-FLD and HPLC-APCI-MS/MS methods. The established new HPLC-FLD method has the advantages of being rapid, simple, selective and sensitive, and could be used for the routine analysis of *P. umbellatus* crude drug.

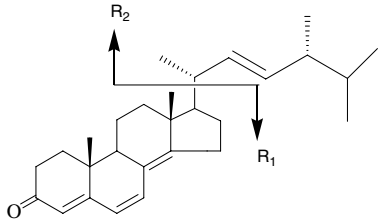
## 1. Introduction

*Polyporus umbellatus*, officially listed in the Chinese Pharmacopoeia, is widely used as a diuretic remedy in Traditional Chinese medicine (Pharmacopoeia of PR China 2005). Many medical prescriptions contain *P. umbellatus*, such as Zhuling Tang, Zhuling Wan, Zhuling San, Wuling San, Fenxiao Tang, Yinchenwuling San, etc. These prescription drugs show excellent efficacy in clinical application, and there have been no reports on its toxicity (Tumor group of Traditional Chinese Medicine Research Institute 1979). *P. umbellatus* is currently used in China as diuretic and antibacterial drug, however diuresis is its main pharmaceutical function (Dictionary of Traditional China Drug).

As reported previously (Miyazaki and Oikawa 1973; Ohsawa et al. 1992; Zheng et al. 2004; Zhou et al. 2007; Sun and Yasukawa 2008), the chemical constituents of *P. umbellatus* mainly involve polysaccharides and steroids. Polysaccharides of *P. umbellatus* have been used as antitumor medicine. Traditional Chinese medicine and contemporary pharmacological studies elucidated that *P. umbellatus* has diuretic activity and is effective in the treatment of some kinds of kidney diseases. However, the chemical structure of diuretic bioactive components have been ambiguous. In 2004, Yuan et al. performed a pharmacological study of steroid compounds in *P. umbellatus* and found that ergosta-4,6,8(14),22-tetraen-3-one-(ergone) was an anti-aldosteronic diuretic bioactive component, its content was determined by high-performance liquid chromatography-ultraviolet detector (HPLC-UV) (Yuan et al. 2003; Yuan et al. 2004). Because of its very low content, the amount of ergone could not be accurately determined by common analytical methods.

In this paper, a high-performance liquid chromatography-fluorescence detector (HPLC-FLD) and high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (HPLC-APCI-MS/MS) approach was described for quantitative analysis of trace ergones in ten batches of *P. umbellatus* from different habitat districts. Compared to HPLC-FLD and HPLC-UV, the HPLC-FLD method was highly sensitive and highly specific. Limit of detection (LOD) was 0.2 μg/mL. Thus, this method could also be used for quantitative analysis of

ergone traces in Chinese Traditional proprietary Medicines. The reliability of the method was further verified by HPLC-APCI-MS/MS. The method was adaptable for evaluation of active compounds in *P. umbellatus*, and was proved to be useful for further study on the relationship between ingredients and bioactivity of *P. umbellatus*. Our studies provided a convenient and reliable analytical method for secondary metabolism and ecology research.




## 2. Investigation, results and discussion

To develop accurate, valid and optimal chromatographic conditions, the different HPLC parameters including mobile phase (methanol-water, methanol-acetone, methanol-tetrahydrofuran, acetonitrile-water or acetonitrile-tetrahydrofuran), category of column (Inertsil ODS-3 column, 5 μm, 250 mm × 4.6 mm i.d., Kromasil C18 column, 5 μm, 250 mm × 4.6 mm i.d., or Diamosil C18 column, 5 μm, 250 mm × 4.6 mm i.d.), column temperature (20, 30, 35 or 40 °C) and flow rate of mobile phase (0.8, 1.0 or 1.2 mL/min) were all examined and compared. For choosing excitation and emission, steady-state visible fluorescence spectra were recorded at room temperature using a HITACHI F-4500 Fluorescence spectrofluorometer. Excitation wavelength varied from 250 to 420 nm and emission wavelength from 400 to 700 nm. Fluorescence spectra of ergone were recorded in methanol at 60 nm/min, with the slits set at 5 nm. Excitation and emission spectra of ergone in methanol are presented in Fig. 1. Finally, an optimized HPLC condition was developed by comparing comprehensively the resolution, baseline, elution time in each chromatogram of different parameters.

The linearity calibration curve for the ergone was assessed at least seven concentration levels range 0.2–35 μg/mL, and triplicate injections were applied at each concentration. calibration curve was constructed by plotting the integrated chromatographic peak areas (Y) versus the corresponding concentration of the injected standard solutions (X). Least square method regression was employed, and the regression equation ($Y = 42.579X - 4.7125$) and coefficient of correlation ($r^2$ was 0.9998) were achieved in relatively wide concentration ranges for all the analyses.

LOD measured based on signal/noise (S/N) $\geq 3$ was 0.2 μg/mL for this assay which was the lowest concentration of the calibration curve. The limit of quantification (LOQ) of this method was set at 0.65 μg/mL measured based on a signal/noise (S/N) ratio $\geq 10$ with acceptable precision (RSD was 9.06%).

**Table 1: Accuracy tests for the proposed HPLC-FLD method**

| Added amount (μg) | Added (μg) | Recovery (%) | Mean recovery (%) | RSD (%) |
|---|---|---|---|---|
| 10 | 9.87 | 98.7 | 100.5 | 2.4 |
| | 10.32 | 103.2 | | |
| | 9.97 | 99.7 | | |
| 30 | 30.6 | 102.0 | 101.2 | 2.5 |
| | 29.5 | 98.3 | | |
| | 31.0 | 103.3 | | |
| 50 | 49.1 | 98.2 | 100.4 | 2.3 |
| | 51.4 | 102.8 | | |
| | 50.1 | 100.2 | | |

The intra- and inter-day precisions were determined by analyzing calibration samples during a single day and on 3 different days, respectively. The RSD values of tests of intra- and inter-day precisions were 2.59% ($n = 3$) and 2.89 % ($n = 3$), respectively. To confirm the repeatability, five different solutions prepared from the same sample were analyzed. The RSD value of the tests of repeatability was 2.51 % ($n = 6$). The recoveries of ergone were also determined by the method of standard addition. Suitable amounts of the reference were spiked into a sample of *P. umbellatus*, which were analyzed previously. The mixture was extracted and analyzed by the proposed procedure. Table 1 shows the results of the tests of accuracy. The results indicate that the RSDs are less than 5%, and the method is acceptable.

The developed HPLC-FLD method was applied for the determination of ergone content in herbal medicine samples. Because of the high sensitivity and selectivity of this fluorescence detection technique, complex matrixes such as herbal extracts could be investigated directly with minimal sample pretreatment. The chromatogram of reference standard and a typical chromatogram obtained from the analyses of extract of *P. umbellatus* are shown in Fig. 2. The peak of ergone (15.4 min) in the herbal extracts was identified by comparing the retention time with its reference compound. The ergone in *P. umbellatus* was determined by the proposed HPLC-FLD method (shown in Fig. 2). The quantitative analyses were performed by means of the external standard method. In some cases, where ergone was present in very low concentration (e.g. ergone in lot no. 079006 and 079012), the samples were concentrated to a proper volume in order to match the linear range. Data of the quantitative analyses by HPLC-FLD method were expressed as mean ± deviation (listed in Table 2). A number of reasons may contribute to the differences in the level of ergone among various samples, such as genetic variation, plant origin, drying process and storage conditions. Variations of this bioactive compound

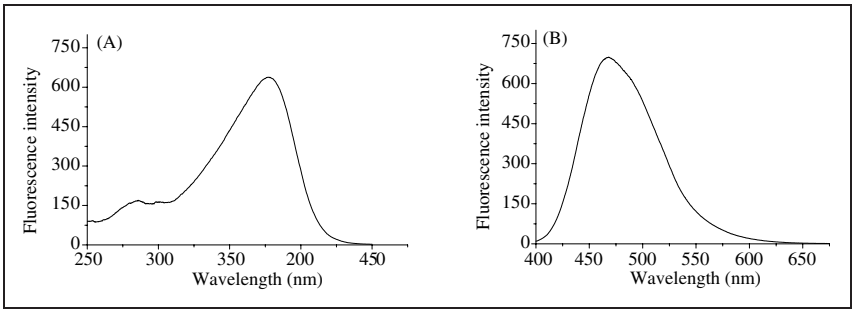



Fig. 1: Fluorescence excitation (A) and emission (B) spectra of ergone in methanol

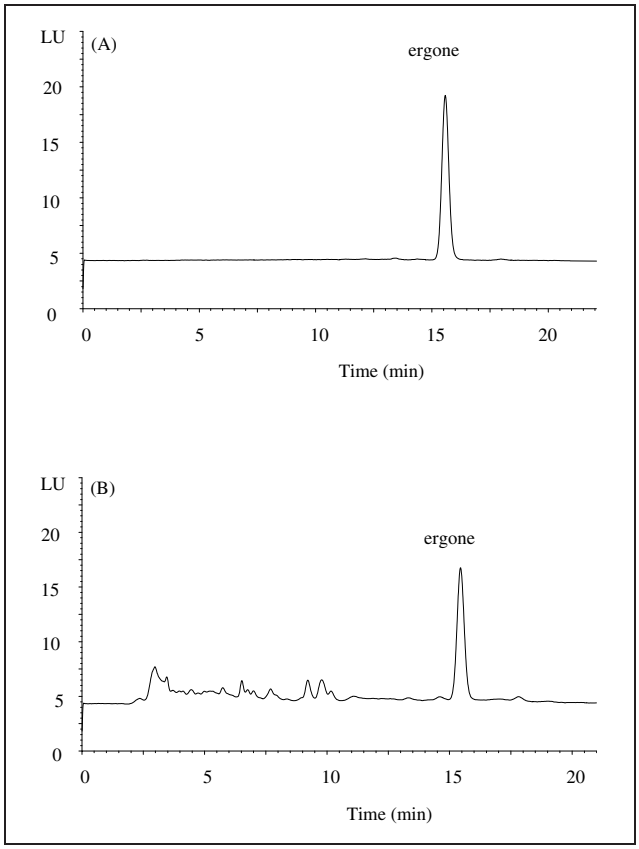



Fig. 2: HPLC-FLD chromatograms of standard compound (A) and typical sample (B)

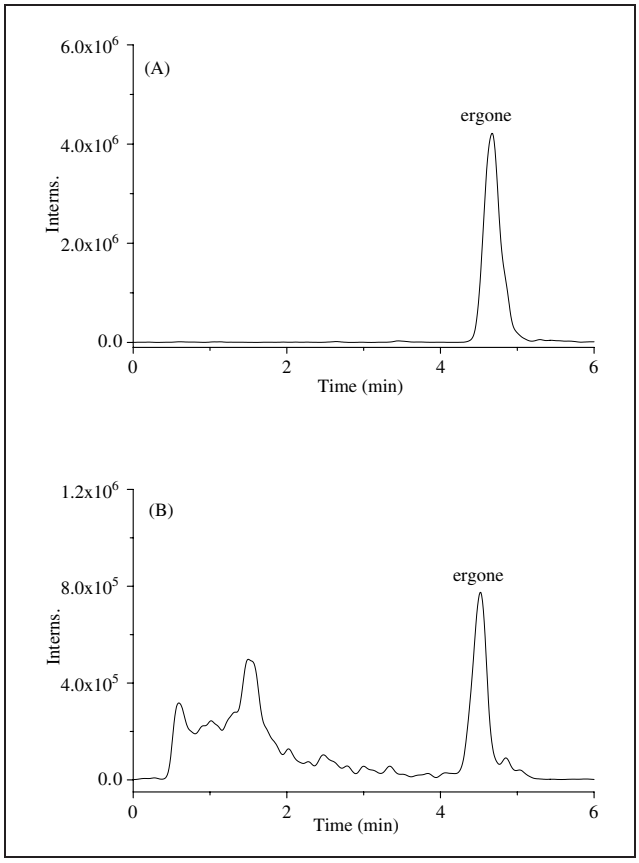



Fig. 3: The total ion chromatograms (TIC) of standard ergone (A) and typical sample (lot no. 079002) (B)

may influence the potencies of *P. umbellatus*. However, the relationship among the quantities of the ergone, their pharmacological activities, and the potencies of *P. umbellatus* needs to be clarified. Further studies on the pharmacological activities of ergone and the potencies of *P. umbellatus* extracts are currently in progress in our laboratory.

In contrast to the previous reported methods in analysis of *P. umbellatus* (Yuan et al. 2003), all the samples were also determined by a HPLC-UV method. The results are shown in Table 2. Compared with the HPLC-FLD method results, the contents of ergone in the samples was determined to be lower by HPLC-UV methods. Because of its very low content, the amount of ergone could not accurately be determined by common analytical methods. In addition, it is difficult to determine ergone by HPLC-UV because of the interference of other chemical compounds.

The newly developed HPLC-FLD method provides much higher specificity, sensitivity, precision and accuracy. By quantification of the ergone, the quality of *P. umbellatus* could be effectively evaluated.

The mass spectrum of ergone was operated in both positive ion and negative ion modes by APCI interface to confirm its molecular weight. Ergone with its carbonyl group were detected with greater sensitivity in a positive ion mode and showed peaks corresponding to $[M+H]^+$. Identification of ergone was clearer in the positive ion mode than in the negative ion mode (spectra not shown). So total ion chromatograms (TIC) from *P. umbellatus* were obtained under the conditions mentioned above (shown in Fig. 3), and the mass spectrum of ergone from *P. umbellatus* was acquired in the positive ion mode (shown in Fig. 4). Retention time of the ergone standard compound and sample are coincident in Fig. 3. Figure 4 exhibits the

**Table 2: Source, obtained and ergone contents (μg/g) of in the ten batches of *P. umbellatus* (mean ± deviation, n = 5)**

| No. | Source | Obtained | HPLC–FLD | HPLC-UV |
|---|---|---|---|---|
| 079002 | Hanzhong, Shaanxi, China | Collected | 9.81 ± 0.03 | 7.54 ± 0.04 |
| 079006 | Hanzhong, Shaanxi, China | Collected | 2.13 ± 0.02 | 2.71 ± 0.04 |
| 079012 | Ningqiang, Shaanxi, China | Collected | 4.05 ± 0.05 | 3.25 ± 0.03 |
| 079013 | Mianyang, Sichuan, China | Collected | 11.73 ± 0.04 | 9.49 ± 0.06 |
| 079014 | Tianshui, Gansu, China | Purchased | 11.91 ± 0.03 | 10.23 ± 0.05 |
| 079015 | Tianshui, Gansu, China | Collected | 9.45 ± 0.06 | 8.97 ± 0.04 |
| 079019 | Xi'an, Shaanxi, China | Collected | 59.17 ± 0.05 | 48.23 ± 0.02 |
| 079020 | Nanyang, Henan, China | Purchased | 14.89 ± 0.06 | 13.58 ± 0.05 |
| 079021 | Mianyang, Sichuan, China | Purchased | 15.71 ± 0.09 | 15.02 ± 0.07 |
| 079022 | Xi'an, Shaanxi, China | Purchased | 5.79 ± 0.04 | 4.98 ± 0.05 |
| Average | | | 14.46 ± 0.05 | 12.40 ± 0.04 |

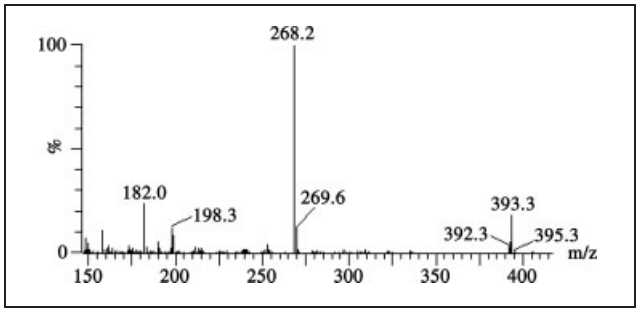



Fig. 4: The typical positive APCI mass spectrum of ergone studied in experiments

intensive protonated molecule of ergone $[M + H]^+$ m/z 393.2, $R_1$ m/z 268 are the main fragment ions of ergone. In $MS^2$ spectra, the fragment ions of losing $R_2$ as positive fragment were observed. Their fragmentation patterns were well matched with their chemical structures.

The performance of the HPLC-FLD method was examined by comparing the ergone concentration with those from HPLC-APCI-MS/MS analysis. Fig. 3 shows a typical extracted SIM chromatogram and MS/MS spectra obtained from the analysis of ergone (m/z 393 → 268) in a spiked standard sample at 10 μg/mL. Herbal samples with relative low, medium and high ergone concentrations were selected for the HPLC-APCI-MS/MS analysis. The results from comparison demonstrated that the two methods gave similar results. For example (lot no. 079002, 079006 and 079012), the ergone concentration determined in *P. umbellatus* by HPLC-APCI-MS/MS was $10.01 \pm 0.04$, $2.03 \pm 0.03$ and $3.93 \pm 0.02$ mg/g, respectively, which was 2.0%, 3.6% and 9.7% different from the HPLC-FLD results. The low detection limit of the HPLC-FLD method allows the identification and quantification of ergone in herbal samples containing very little ergone.

## 3. Experimental

### 3.1. Reagents and materials

HPLC-grade methanol was purchased from Fisher Company (fisher Inc., USA). Ultrapure water was prepared by a Millipore SAS 67120 MOLSHEIM (France). The standard of ergone was isolated by the author from the sclerotia of *P. umbellatus*. Its structure was characterized by chemical and spectroscopic methods ($^1$H NMR, $^{13}$C NMR, 2D NMR, MS, UV and IR) and compared with the literature (Tanaka et al. 1996; Lee et al. 2005). Purity analysis showed that its purity was above 99% (HPLC). Ten samples of *P. umbellatus* were collected or purchased from different regions of China (shown in Table 2), and they were authenticated by Professor Yazhou Wang (College of Life Sciences, Northwest University, Xi'an, Shaanxi, China ). Voucher specimens (079014) were deposited in Biomedicine Key Laboratory of Shaanxi Province, Northwest University, Xi'an, Shaanxi, P. R. China.

### 3.2. Preparation of standard solutions

The reference standard of ergone was accurately weighed, dissolved in methanol and diluted to appropriate concentration. Stock solution of the ergone (40 μg/mL) was prepared in methanol. The stock solution was further diluted to make working solutions. All the solutions were stored in the refrigerator at 4 °C before analysis.

### 3.3. Preparation of samples

*P. umbellatus* was dried at 50 °C until constant weight. Each dried material was pulverized to 80 meshes. Pulverized powder (1 g) was accurately weighed and then ultrasonically extracted with 20 mL of methanol for 1 h. The supernatant solution was filtrated, then cooled to ambient temperature. The solution was evaporated to dryness under reduced pressure and the residue was dissolved in 2 mL of methanol. The obtained solution was filtered through a syringe filter (0.45 μm) and aliquots (20 μl) were subjected to HPLC analysis.

### 3.4. HPLC-FLD analysis

An Agilent-1100 HPLC system with ultraviolet detector and fluorescence detector (HPLC-UV-FLD) was used (Agilent Corporation, MA, USA) for quantitative determination. The chromatographic separation was performed on an Inertsil ODS-3 analytical column (5 μm, 250 mm × 4.6 mm i.d., Japan) with the column temperature set at 30 °C. An isocratic elution of methanol (A) and water (B) was 98 : 2 (v/v) in 30 min. The flow rate was 1.0 mL/min, and the injection volume was 20 μL. Fluorescence detector has excitation wavelength at 380 nm and emission wavelength at 468 nm. Data acquisition was performed using software Chemstation (Agilent Corporation, MA, USA).

### 3.5. Calibration curve, limits of detection (LOD) and quantification (LOQ)

Methanol stock solution was prepared and diluted to appropriate concentrations for the construction of a calibration curve. Seven concentrations of solution were injected in triplicate, and the calibration curve was constructed by plotting the value of peak areas versus the value of concentrations of each analyte. LOD and LOQ under the present chromatographic conditions were determined on the basis of response and slope of each regression equation at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

### 3.6. Precision, repeatability and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed method. The intra-day variation was determined by analyzing the same mixed standard solution six times within one day. While for the inter-day variability test, the solution was examined in triplicate for consecutive 3 days. The RSD of the retention time and peak area were taken as the measures of precision. To confirm the repeatability, five different working solutions prepared from the sample No. 079012 were analyzed. The RSD was taken as the measures of repeatability.

Recovery test was used to evaluate the accuracy of this method. For determination of the recoveries of analyses in pretreatment of crude drugs, three different concentration levels of final purified extracts were submitted to all extraction, filtration and purification steps as described in Section 3.3. The average recovery was determined by the formula: recovery (%) = (observed amount-original amount/spiked amount) × 100%, and RSD (%) = (SD/mean) × 100%.

### 3.7. HPLC-APCI-MS/MS analysis

HPLC-APCI-MS/MS analyses were performed using a Waters 2996 liquid chromatography system (Waters Corporation, USA) with a Waters symmetry C18 column, 5 μm, 50 mm × 2.1 mm i.d. (Waters Corporation, USA). The mobile phase was methanol-water (98 : 2, v/v) at a flow rate of 0.4 mL/min. The column temperature was maintained at 30 °C. A Waters Quattro premier XE mass spectrometer equipped with an atmospheric pressure chemical ionization source was set with a drying gas ($N_2$) flow of 150 L/h. The voltage of capillary, extractor and RF lens was set at 3.5 kV, 4 and 0.8 V, respectively. The temperature was maintained at 120 and 205 °C for source and desolvation, respectively. The gas flow rate for desolvation and cone was set at 150 and 100 L/h, respectively. The full scan mass spectrum was acquired over range of m/z 100–600. The cone voltage for ergone was at 50. HPLC-APCI-MS/MS was performed in selected ions monitoring (SIM) using target ions at $[M + H]^+$ m/z 393.2 for ergone. Data acquisition was performed using Masslynx 4.1 software (Waters Corporation).

Acknowledgement: This work was financially supported by the State Pharmacopoeia Commission of PR China, Pharmacopoeia of the People's Republic of China (No. YZ–199∼200).

**References**

Jiangsu New Medical College (1985) Dictionary of Traditional China Drug, Shanghai Science and Technology Press, Shanghai, p. 2191–2193.

Lee WY, Park Y, Ahn JK, Park SY, Lee HJ (2005) Cytotoxic activity of ergosta-4,6,8(14),22-tetraen-3-one from the sclerotia of Polyporus umbellatus. Bull Korean Chem Soc 26: 1464–1466.

Miyazaki T, Oikawa N (1973) Studies on fungal polysaccharide. XII. Water-soluble polysaccharide of Grifora umbellata (Fr.). Chem Pharm Bull 21: 2545–2548.

Ohsawa T, Yukawa M, Takao C, Murayama M, Bando H (1992) Studies on constituents of fruit body of Polyporus umbellatus and their cytotoxic activity. Chem Pharm Bull 40: 143–147.

Sun Y, Yasukawa K (2008) New anti-inflammatory ergostane-type ecdysteroids from the sclerotium of Polyporus umbellatus. Med Chem Lett 18: 3417–3420.

Tanaka N, Hosoi KI, Tanaka D, Takahashi M (1996) Photochemical reaction of ergosta-4,6,8(14),22-tetraen-3-one. Chem Pharm Bull 44: 843–846.

The State Pharmacopoeia Commission of PR China, Pharmacopoeia of the People's Republic of China, vol.1, Chemical Industry Press, Beijing, 2005, p. 222.

Tumor groups of Traditional chinese medicine Research Institute (1979) Toxic experimental observation of polyorus umbellatus extract. J New Med 5: 315–317.

Yuan D, Mori J, Komatsu KI, Makino T, Kano Y (2004) An anti-aldosteronic diuretic component (drain dampness) in Polyporus sclerotium Chem Pharm Bull 27: 867–870.

Yuan D, Yamamoto KI, Bi KS, Zhang P, Liu F, Kano Y (2003) Studies on the marker compounds for standardization of Traditional Chinese Medicine "Polyporus Sclerotium". Yakugaku Zasshi 123: 53–62.

Zheng SZ, Yang HP, Ma XM, Shen XW (2004) Two new polyporusterones from polyporus umbellatus. Nat Prod Res 18: 403–407.

Zhou WW, Lin WH, Guo SX (2007) Two new polyporusterones isolated from the sclerotia of Polyporus umbellatus. Chem Pharm Bull 55: 1148–1150.